

# Histopathological effects of acute exposure to nickel oxide nanoparticles in the aquatic gastropod *Physa acuta*

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Recursos Biológicos Aquáticos

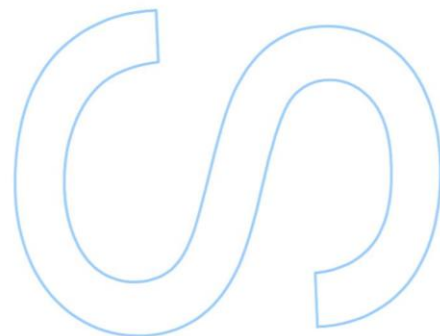
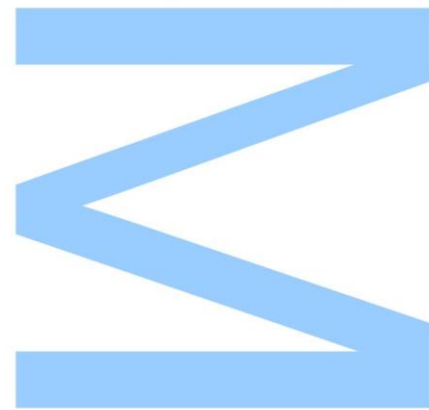
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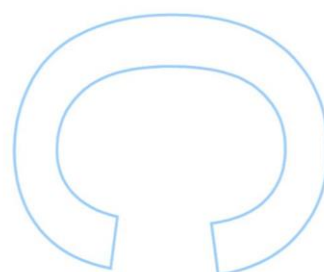
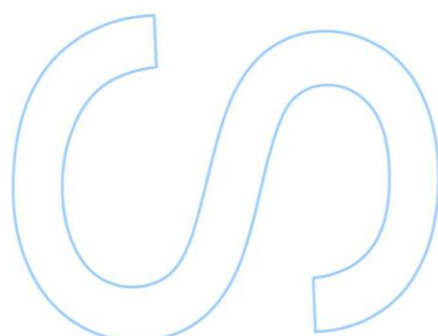
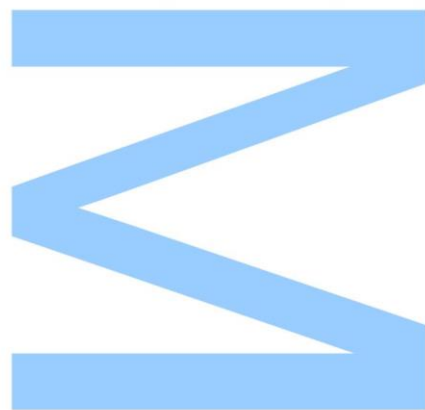




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, \_\_\_\_/\_\_\_\_/\_\_\_\_



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*À memória de meu irmão,*

*“You can climb a ladder up to the sun*

*Or write a song nobody has sung*

*Or do something that's never been done,*

*Do something that's never been done”*

*(Coldplay)*

## Abstract

The advances made in nanoscience and nanotechnology in the last decades have increased the presence in the environment of engineered nanoparticles, which are considered an emerging contaminant. Nanoparticles unique physical and chemical characteristics make them a product capable of affecting a myriad of organisms. They may also constitute a risk for human health by entering the trophic chain. In fact, the nanoparticles impact on the environment, aquatic organisms and human health are yet little known and further studies with innovative tools are necessary for the evaluation of the effects of toxicity.

Nickel oxide nanoparticles have attracted increasing attention due to the potential use in a variety of applications. Developments in science and technology are increasing the quantity of products with nickel oxide nanoparticles and this trend will probably continue. Therefore, the environmental contamination with nickel oxide nanoparticles and consequent effects in the aquatic ecosystems are undeniable.

*Physa acuta* is an invasive freshwater gastropod that has now a cosmopolitan distribution. It is a small deposit feeder and R-selected hermaphroditic species with rapid maturation and high reproductive rate. These characteristics allied with its ease of culture make it an excellent biological research model.

The aim of this study was to assess the effects of the acute exposure to oxide nickel nanoparticles in the aquatic gastropod *Physa acuta* using histology as biomarker.

With this purpose, two sequential acute bioassays with nickel oxide engineered nanoparticles with the snail *Physa acuta* were performed. The concentrations of contaminant tested were 2.7, 4.7, 8.2, 14.4, 25.2 mg/L and 21.3, 37.3, 65.3, 114.3, 200.0 mg/L. The samples subjected to concentrations corresponding to NOEC and LOEC were histologically processed using standard techniques. The histopathologies found were scored with a semi-quantitative method based on the extension of the lesions. The average of the extension of histological responses observed were calculated for each organ and tissue.

In this study it was stated that the histology of several *Physa acuta* organs were affected by nickel oxide engineered nanoparticles, even in sub-lethal concentrations. All histopathological effects were observed in NOEC and LOEC. However, the extension of the lesions was dose dependent in most of the cases, this is in the generalized inflammatory response detected in snail tissues, the lesions observed in the foot and in the ganglia nervous tissue. The extension of histopathological changes observed in the hermaphrodite gland were significantly different from the control only at high levels of

NiO ENPs, this is at LOEC. In opposition, the extension of histopathological lesions observed in the glands from the foregut located near radula are not significantly different in NOEC and LOEC and even in the highest concentration the glands were not extensively affected.

Histology demonstrated to be a promising tool as a biomarker of toxicity for studies with engineered nanoparticles in the aquatic environment, as well as the use of the aquatic gastropod *Physa acuta*. It is expected that these results will contribute to the understanding of the toxic effects of engineered nanoparticles in the aquatic environment and help to establish protocols for control and protection of the aquatic organisms.

## Keywords

Nickel oxide nanoparticles, engineered nanoparticles, *Physa acuta*, ecotoxicity, histology, histopathology, biomarkers.

## Resumo

Os avanços alcançados na última década nas áreas da nanociência e nanotecnologia têm provocado um aumento da ocorrência e quantidade de nanopartículas manufaturadas no meio ambiente, levando a que estas sejam consideradas um contaminante emergente. As características físicas e químicas únicas das nanopartículas conferem-lhes o potencial para afetar uma miríade de organismos. Adicionalmente, podem constituir um risco acrescido à saúde humana ao entrarem na cadeia trófica. Os efeitos das nanopartículas manufaturadas no ambiente e nos organismos aquáticos, assim como as suas consequências para a saúde humana são ainda pouco conhecidos e, por essa razão, são necessários estudos, com ferramentas inovadoras, para avaliar o potencial toxicológico destes compostos.

As nanopartículas de óxido de níquel têm atraído bastante atenção devido ao seu enorme potencial de aplicações. A crescente utilização destes produtos, está a aumentar a quantidade de nanopartículas de óxido de níquel no ambiente e esta tendência irá continuar nos próximos anos. Assim, a contaminação dos ecossistemas aquáticos com estas partículas e as consequências associadas são inevitáveis.

*Physa acuta* é um gastrópode aquático de água doce. É uma espécie invasora que se encontra, atualmente, distribuída mundialmente. Alimentam-se de detritos sedimentados e são hermafroditas com seleção tipo R. A sua rápida maturação e as elevadas taxas reprodutivas, aliadas ao fácil cultivo em laboratório, fazem com que *P. acuta* seja um excelente modelo biológico.

O objetivo deste estudo foi avaliar os efeitos toxicológicos da exposição aguda a nanopartículas de óxido de níquel no gastrópode aquático *Physa acuta*, usando a histologia como biomarcador.

Com este propósito, foram realizados dois ensaios sequenciais de toxicidade aguda com nanopartículas manufaturadas de óxido de níquel e o caracol aquático *Physa acuta*. As concentrações testadas foram 2.7, 4.7, 8.2, 14.4, 25.2 mg/L e 21.3, 37.3, 65.3, 114.3, 200.0 mg/L. As amostras sujeitas a concentrações correspondentes ao NOEC e LOEC foram selecionadas para a técnica histológica de rotina. As histopatologias encontradas foram classificadas com um método semiquantitativo baseado na extensão das lesões. A média da extensão das respostas histológicas foi calculada para cada órgão ou tecido.

Os resultados obtidos indicam claramente que as nanopartículas de óxido de níquel provocam alterações histológicas nos órgãos de *Physa acuta*, mesmo em concentrações subletais. Todas os efeitos histopatológicos foram observados nas concentrações correspondentes ao NOEC e LOEC. Contudo, na maioria dos casos a extensão das lesões histopatológicas foi dose-dependente, isto é, na resposta inflamatória generalizada, nas lesões do pé e nos tecidos nervosos ganglionares. As extensões das lesões histopatológicas observadas na glândula hermafrodita foram significativamente diferentes do controlo apenas para níveis elevados de NiO ENPs, isto é, no LOEC. Pelo contrário, as extensões das lesões histopatológicas observadas nas glândulas da porção anterior do tubo digestivo localizadas junto à rádula não apresentaram diferenças significativas entre o NOEC e LOEC, mais ainda, mesmo na concentração mais elevada estas glândulas não estavam extensivamente afetadas.

A análise histológica demonstrou ser uma ferramenta promissora como biomarcador de toxicidade para estudos com nanopartículas no ambiente aquático, bem como o uso do gastrópode aquático *Physa acuta*. É esperado que estes resultados contribuam para a compreensão dos efeitos toxicológicos das nanopartículas manufaturadas no ambiente aquático e que ajudem a estabelecer novos protocolos de controle e de proteção dos organismos aquáticos.

## Palavras-chave

Nanopartículas de óxido de níquel, nanopartículas manufaturadas, *Physa acuta*, ecotoxicidade, histologia, histopatologia, biomarcadores.



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## LIST OF ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic acid
ENPs	Engineered nanoparticles
EU	European Union
HE	Haematoxylin eosin
INPs	Incidental nanoparticles
LOEC	Lower observed effect concentration
Ni	Nickel
NiO	Nickel oxide
NNPs	Natural occurring nanoparticles
NOEC	No observed effect concentration
NPs	Nanoparticles
ROS	Reactive oxygen species

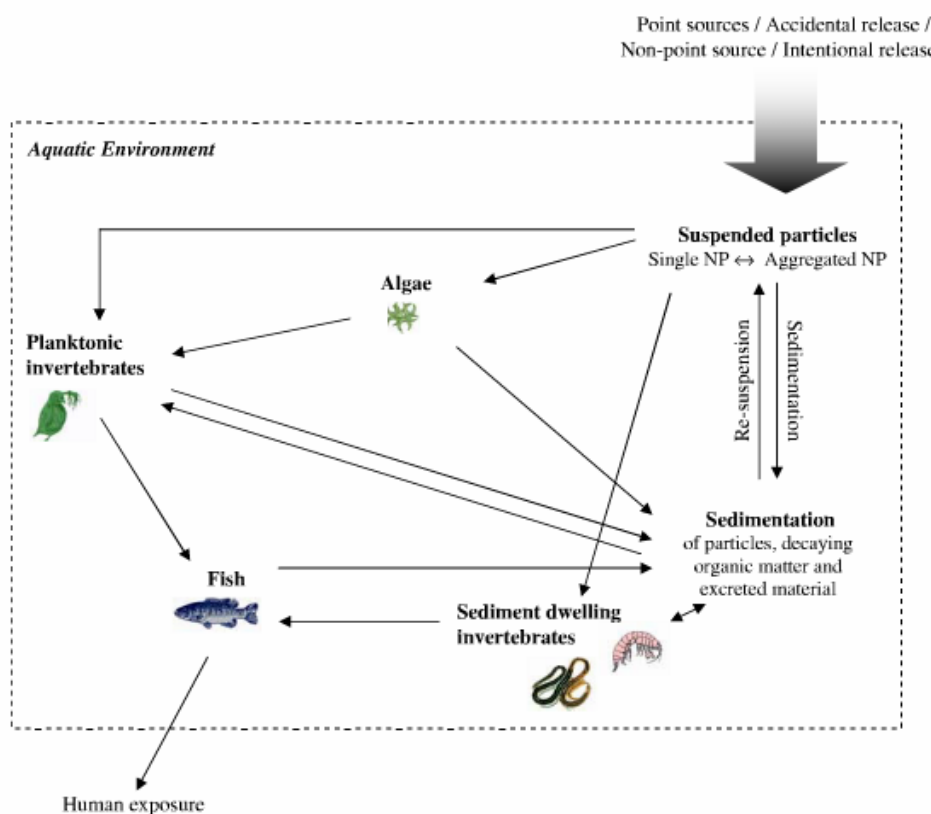
# 1. INTRODUCTION

## 1.1. Nanoparticles

Nanoparticles (NPs) are nano-objects with all three external dimensions with sizes between 1 and 100 nanometres (ISO, 2017) which are produced by a diversity of chemical and physical processes (Din & Rani, 2016). They are classified as naturally occurring (NNPs), incidental (INPs) or engineered (ENPs) depending if they are the result of natural processes, by-products of human activities or intentionally synthesised by men, respectively. ENPs are controlled in shape and chemical composition. They have large areas per unit of volume and novel electronic and chemical properties when compared with the same elements in a bulk form (Lead & Smith, 2009; Lohse, 2013; Morris & Willis, 2007).

Recently, nanoscience and nanotechnology have made great advances, and ENPs have been increasingly incorporated in a wide variety of products and used in a wide range of applications such as environmental remediation, pollution sensors, electronic appliances, photovoltaics products, automotive industry, medical imaging apparatus, drug delivery systems, clinical endodontics, cosmetics and hygiene products (Dowling, 2004; EPA, 2010; Matoba & Egashira, 2014; Shrestha & Kishen, 2016; Vance et al., 2015). The increasing quantity of ENPs in several environmental compartments and the awareness of the potential consequences made them a major concern, especially when it comes to aquatic pollution (Lead & Smith, 2009). Consequently, ENPs are considered an emerging contaminant (EPA, 2010; Richardson & Kimura, 2015; Sauvé & Desrosiers, 2014).

In the aquatic environment, ENPs can be in suspension, sediment (in single or in aggregates forms) or resuspend and even be dispersed to long distances. ENPs can enter the biological systems of aquatic organisms via ingestion and via external surface epithelia (integument and respiratory system). At a cellular level, internalization may occur through various processes (e.g. endocytosis), potentially leading to various types of toxic cell injury (Moore, 2006). Also, they can affect a myriad of aquatic organisms, affect humans by entering the trophic chain and may constitute other risks, since they interact with the medium in uncertain ways (Fig. 1) (Baun et al., 2008).



**Fig. 1.** Potential pathways for nanoparticles contaminants in the aquatic environment. Source: Baun et al., (2008).

The unique characteristics listed above and the small size of ENPs makes the quantification of these materials quite challenging, and the environmental analyses often requires the use of multiple technologies (EPA, 2010). In fact, ENPs impacts on the environment, aquatic organisms and human health are yet little known and further studies with innovative tools are necessary for the evaluation of its possible toxic effects (Fabrega et al., 2011; Gavrilescu et al., 2015; Moore, 2006). Understanding the potential risks and hazards and the biological responses associated with ENPs will make it possible to prevent or mitigate such effects.

## 1.2. Nickel and nickel oxide nanoparticles

Nickel is a transition metal with classically metallic properties. It is a good conductor of both heat and electricity, it is ferromagnetic and has many applications (e.g., electroplating, battery production, to form alloys with other metals). It can exist in several oxidation states, the more abundant and significant being the +2 oxidation state, such as NiO. Although Ni is an essential trace element in animals, it is graded as very toxic

(ATSDR, 2005). The toxic effects of Ni in its bulk form are well documented and extend from plants to animals (Das et al., 2008; Denkhau & Salnikow, 2002; Seregin & Kozhevnikova, 2006). Moreover, in the EU, the Water Framework Directive 2000/60/EC (which establishes a common action in the field of water policy, a strategy against the pollution of the Community waters and foresee the identification of priority substances which represent a significant risk to, or via, the aquatic environment at Union level) contemplates Nickel and its compounds in the list of priority substances of the UE under the Environmental Quality Standards Directive 2008/105/EC and in appendix X of the Directive 2013/39/EU amending Directives 2000/60/EC and 2008/105/EC (EU, 2000; 2008; 2013).

Nickel Oxide (NiO) ENPs have attracted increasing attention due to their potential use in a variety of applications. NiO ENPs can be applied as catalysts, battery cathodes, anti-ferromagnetic layers, nanowires, nanofibers, specific alloys, ceramic structures, automotive rear-view mirrors with adjustable reflectance, gas sensors, electrochromic films, magnetic materials, among others (AZoNano, 2013). NiO ENPs have various manufacturing possibilities and the additional benefit of being a low-cost ion storage material (Din & Rani, 2016; El-Kemary et al., 2013; Trung et al., 2015). More recently, in the field of biomedical engineering they have been successfully used in the development of diagnosis tools (Atta et al., 2016). NiO ENPs appear like a green powder and are graded as very toxic. They can cause an allergic skin reaction, prolonged harmful effects to aquatic life, and possible damage to organs in prolonged or repeated exposure (AZoNano, 2013). Since developments in science and technology are increasing the quantity of products with NiO ENPs and this trend will probably continue, the environmental contamination with NiO ENPs and consequent effects in the aquatic ecosystems are undeniable.

### 1.3. The freshwater gastropod *Physa acuta* as a biological research model in aquatic toxicology

The snail *Physa acuta* Draparnaud, 1805 (Fig. 2) is an invasive freshwater gastropod that has now a cosmopolitan distribution (Ng et al., 2015). It is a small, left-handed, airbreathing snail that lives in both lentic and lotic waters, although their populations reach maximum densities in lentic waters. It is a deposit feeder, grazing periphyton and organic detritus that accumulates on rocky substrata or even large aquatic plants. *P. acuta* is an R-selected species with rapid maturation and high reproductive rate. It is a

relatively easy species to culture under controlled conditions and laboratory populations mature in 6 – 8 weeks. These snails are hermaphroditic and prefer to outcross, but self-fertilization occurs successfully when specimens are isolated. Male maturity arrives slightly before than female one. After maturation, each adult can lay 50 to 100 eggs weekly thereafter for up to a year.



**Fig. 2.** The gastropod *Physa acuta*. Source: David Liebman in Dillon et al. (2006).

*P. acuta* is an excellent biological model to perform bioassays on life history, behaviour, competition, predation and toxicology (Batley & Simpson, 2016; Dillon et al., 2006). According to Musee et al. (2010) the high abundance and biomass, the benthic locomotion and feeding habits (vulnerability to elevated concentrations of pollutants in sediment) and the importance of *P. acuta* in the food chain of many freshwater organisms make this species a good candidate for ecotoxicological studies on ENPs. However, the use of aquatic snails requires more skilled handling by laboratory staff and its manipulation becomes increasingly time-consuming, especially when dissection and shell removing is necessary (Sokolowski et al., 2003).

Furthermore, being an invertebrate, *P. acuta* has a much lower degree of neurophysiological sensitivity when compared with vertebrates. This complies with article 13 of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes regarding the choice of methods that states: “shall be selected animals with the lowest capacity to experience pain, suffering, distress or lasting harm” (EU, 2010).



## 1.4. Supporting studies

The potential contamination of the environment with ENPs increased the attention of the scientific community to these compounds. As a result, many studies have been conducted to evaluate their effects on living organisms.

Several studies on human aortic endothelial cell lines showed that ENPs can elicit a pronounced inflammatory response, cytotoxic injury and lead to cell death, although these results depend on the concentration and composition of the ENPs (Gojova et al., 2007; Gojova et al., 2009; Kennedy et al., 2009). Similarly, assays in human lung cell lines and rat lungs conducted by Lu et al. (2009) showed the potential for ENPs, including NiO ENPs, to generate significant intrinsic free radical activity, cytotoxicity and inflammogenic responses. Hussain et al. (2005) research in rat liver cell lines suggested that the acute toxic effects of metal/metal oxide ENPs led to the accumulation of reactive oxygen species through depletion of glutathione (cell antioxidant) levels in the mitochondrial membrane.

In a review, Shaw & Handy (2011) concluded that the adverse physiological effects in fish and the hazard of some metal ENPs are different from the ones produced by traditional dissolved forms (non nano-scale). Cedervall et al. (2012) studied the transport of polystyrene ENPs in the aquatic food chain from algae, through zooplankton to fish, and observed that the ENPs affect the lipid metabolism and behaviour of the top consumer. Musee et al. (2010) showed that ENPs ( $\alpha$ -alumina and  $\gamma$ -alumina) can affect the embryonic growth and hatchability on *P. acuta*, even when exposed to sublethal concentrations. In this case ENPs caused irregular egg masses with less eggs, aberrant embryos and slower embryonic development.

Many gastropod organisms have showed to be a suitable research model, particularly in toxicity tests. Moreover, a few toxicological studies on *Physa* with others chemical compounds (apart from NPs) used histology as a biomarker, successfully (Bacchetta et al., 2002; Roses et al., 1999; López-Doval et al. 2014).

Chemical contaminants can affect biological systems at many levels, but they all start by changing structural or functional properties of molecules essential to cellular activities, altering the structure or function of organelles and cells. These changes alter the physiological state of the organisms which can affect growth and reproduction. This cascade of events can ultimately result in changes of populations dynamics and may affect the community structure (Newman & Jagoe, 1996). The advantage of the use of

histology as a biomarker lies in its intermediate location in the hierarchy of biological organization (Bernet et al., 1999; Saraiva et al., 2015). Thus, the use of this biomarker appears to be an excellent tool to study the changes caused by ENPs at cellular and tissue levels.

As above mentioned, despite the potential advantages of using aquatic gastropods in the assessment of ENPs toxicity, studies on the effects of these compounds in these organisms are very scarce. Moreover, to the best of our knowledge, there aren't any studies on the histopathological effects of ENPs in aquatic snails. Therefore, the aim of the present study was to assess the histopathological effects of the acute exposure to oxide nickel nanoparticles in the aquatic gastropod *Physa acuta*, also considering the potential usefulness of histology as a biomarker of toxicity.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Animals used in the experiments were selected from a stock of laboratory-reared snails (*Physa acuta*) with the same age and a similar size (mean shell length  $5.6 \pm 0.5$  mm; mean fresh weight  $24.3 \pm 6.27$  mg), originated from egg clusters spawned at the same time. Animals were acclimatized to the medium, temperature and photoperiod of experimental conditions for 72 h prior the bioassays.

### 2.2. Nanoparticles

NiO ENPs (nearly spherical with a particle size of 100 nm, 99% purity), were obtained from Nanostructured & Amorphous Materials Inc. (Houston, TX, USA). A stock suspension of 200 mg/L NiO ENPs was prepared by stirring ENPs in ASTM medium (artificial hard freshwater) for half an hour. ASTM medium was prepared according to ASTM (1980).

### 2.3. Toxicity bioassays

Bioassays were performed in 250 mL beakers with 100 mL of ASTM medium, to which 50 mL of sterile fine sand were added as a substrate. Beakers were left standing

24 h for the complete sedimentation of sand. Then a defined volume of a NiO ENPs stock suspension (in ASTM; to obtain the required concentrations of ENPs) were introduced and left to settle and stabilize for 48 hours. Following, 10 snails were carefully introduced in each beaker, avoiding substrate resuspension. Two independent sequential bioassays were conducted, differing in the concentration of NiO ENPs: in the first bioassay 2.7, 4.7, 8.2, 14.4, 25.2 mg/L were tested; in the second bioassay 21.3, 37.3, 65.3, 114.3, 200.0 mg/L were tested. These concentrations are within the range of ENPs that can be found in the environment (Musee et al., 2010). In both bioassays, the dilution factor was 1.75.

All concentrations and a control without ENPs were tested in triplicate. Beakers were incubated for 96 h in a thermoregulated chamber at  $20 \pm 1^\circ\text{C}$ , under a photoperiod of 12 h light/12 h dark. Snails were kept unfed and the medium was not renewed during the incubation period. Mortality was set as the endpoint: at the end of the bioassays, all snails were inspected under a stereomicroscope and death was confirmed by the absence of movements. Fifteen surviving snails (five per replicate;  $n=15$ ) were sampled from the highest sublethal concentration (NOEC), from the lowest lethal concentration (LOEC) and from the control for histological analysis.

## 2.4. Histological analysis

The snails (the whole organism) were fixed in 10% buffered formalin (1:10 vv) during 48 h, then transferred to 70% ethanol.

Prior to the processing of the definitive samples, a method optimization was performed to either soften or remove the snail shell. The following methods were tested: processing the organism with the shell; decalcification of the shell with ethylenediaminetetraacetic acid (EDTA); removal of the shell with tweezers (included cleaning the sand trapped in the mucous); and removal of the shell and cleaning the sand, followed by 72 h EDTA decalcification and dissection of the posterior intestine for removal of ingested sand fragments.

All samples were processed using standard techniques in an automatic tissue processor (Appendix I) and each snail was sagittally cut into 5  $\mu\text{m}$  thickness sections and stained with hematoxylin and eosin (HE) (Appendix II). Four sections of each snail were observed and histological changes were identified and registered.

In the absence of a quantitative or semi-quantitative method to classify the histopathological lesions of gastropods, similar to the existent for fish (Bernet et al., 1999), a semi-quantitative method based on the extension of the lesions was created (Table 1). Histopathological lesions were scored with this method and the average of the extension of observed histological changes was calculated for each organ or tissue.

**Table 1**

Semi-quantitative method used to classify the extension of histopathological changes found in the snail *Physa acuta*.

Scale	Extension of lesion
0	Unchanged
1	Mild (<33% tissue affected)
2	Moderate ( $\geq 33\%$ and $< 66\%$ tissue affected)
3	Severe/Diffuse ( $\geq 66\%$ tissue affected)

## 2.5 Data analysis

All data were analyzed using IBM SPSS Statistics 23 software. The increase of the extension of histopathological changes on the organs and tissues of snails exposed to increasing NiO ENPs concentrations was evaluated using the non-parametric Jonckheere-Terpstra test, followed by pairwise comparisons whenever statistical significance was detected. Statistical significance was accepted when  $p < 0.05$ .

## 3. RESULTS

### 3.1. Toxicity bioassays

No mortality was registered in the first bioassay. In the second bioassay, the NiO ENPs concentrations corresponding to NOEC and LOEC were 65.3 mg/L and 144.3 mg/L, respectively. The mortality rates in LOEC were 10%, 40% and 20% in each replicate, respectively. Furthermore, 200.0 mg/L of NiO ENPs caused 100% mortality rate (Table 2). No behavioural alterations were observed in both bioassays.

**Table 2**

Mortality rate (%) of *Physa acuta* snails in the second bioassay. The concentrations 0.0, 65.3 and 114.3 mg/L represent control, NOEC and LOEC, respectively.

Replicate	Concentrations (mg/L)					
	0.0	21.3	37.3	65.3	114.3	200.0
1	0	0	0	0	10	100
2	0	0	0	0	40	100
3	0	0	0	0	20	100

## 3.2. Histological analysis

### 3.2.1. Method optimization

Shell removal with tweezers (included cleaning the sand trapped in the mucous), followed by 72h EDTA decalcification and dissection of the posterior intestine for removal of ingested sand fragments was the method that produced better results in the sectioning of the whole organism and was subsequently used in the definitive samples. All other methods tested caused cuts or rips in the paraffin sections, probably due to the sand fragments either in the intestine or in the interior of the shell.

### 3.2.2. Histopathology

Although all the organs were observed, some of them showed no evident histological changes due to NPs exposure and therefore were not selected for further analysis. Moreover, some other organs were also rejected due to difficulties in the fixation technique. Evident histological changes, that can be useful as potential biomarkers, were detected in the following organs and tissues: ganglia nervous tissues, reproductive system in the hermaphroditic gland, foot tissues and glands from the foregut. A general inflammatory response in the connective tissues of the whole organism, characterized by an increase of pigment deposits and haemocyte infiltration, was also observed in exposed snails and included in the analysis.

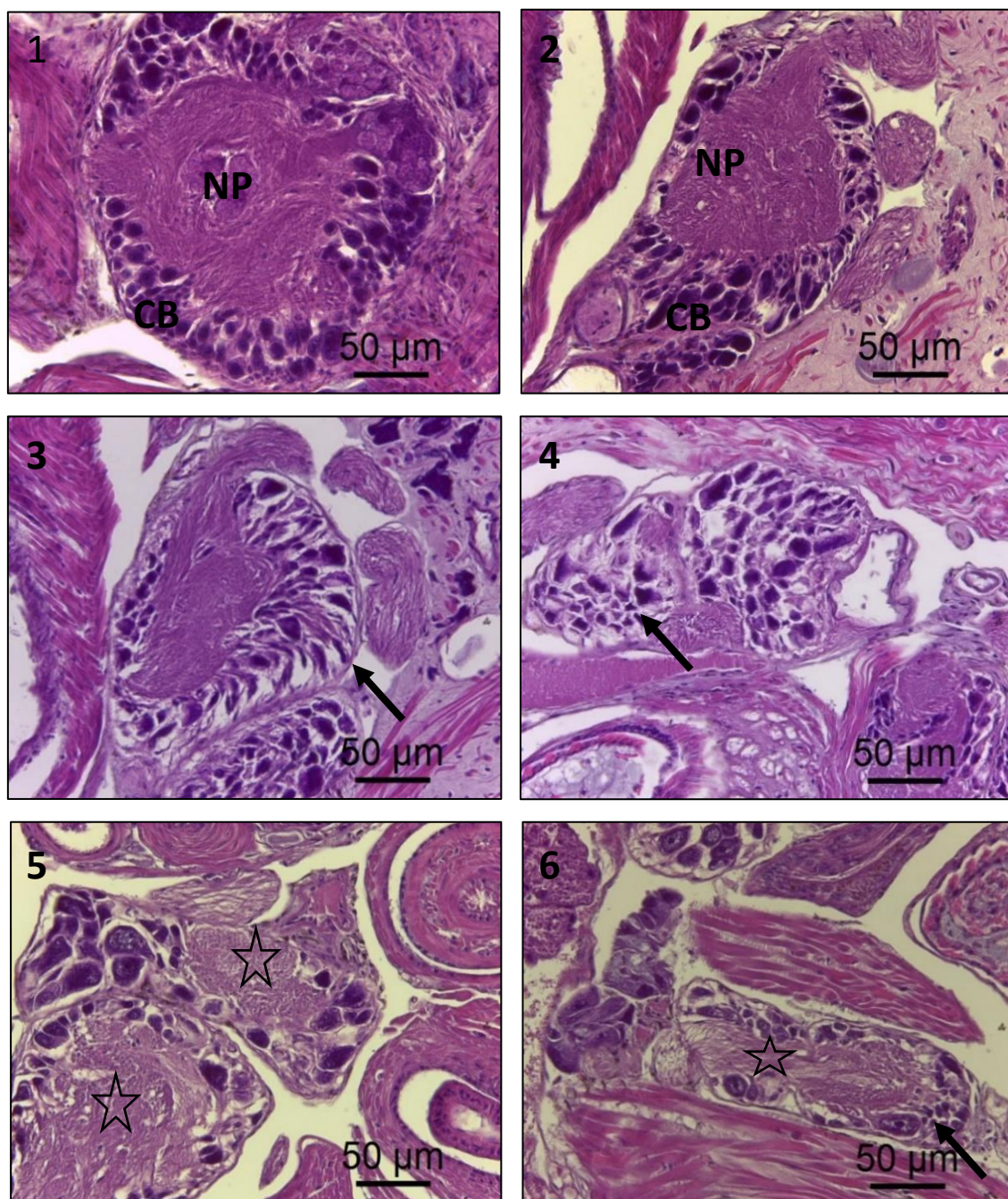
The variation of the extension of all the histopathologies found and distribution of affected snails for each class are shown in Appendix III.

## Ganglia nervous tissues

Histologically, the ganglia are enveloped into a connective tissue capsule, with the cell bodies of neurons on the periphery and the neuropil in the central part of the ganglion (Fig. 3.1; 3.2). The histological response of these tissues to the NiO ENPs was the most severe and the extension of the lesions was dose dependent and (Table 3).

The histological changes observed were neuropil degeneration (Fig. 3.5; 3.6) (characterized by shrinkage, disruption and axonic structural loss) and necrosis of neurons cell bodies (Fig. 3.3; 3.4; 3.6).

Neuropil degeneration was observed more frequently and extended as NiO ENPs concentration increased, with no snails diffusely affected (class 3) in NOEC, as oppose to the high percentage of snails diffusely affected in LOEC (Appendix III – Fig.7). Likewise, necrosis of neurons cell bodies also increased in extension and frequency as the NiO ENPs concentration increased, though the classification severe/diffuse (class 3) occurred also in NOEC (Appendix III – Fig. 7).



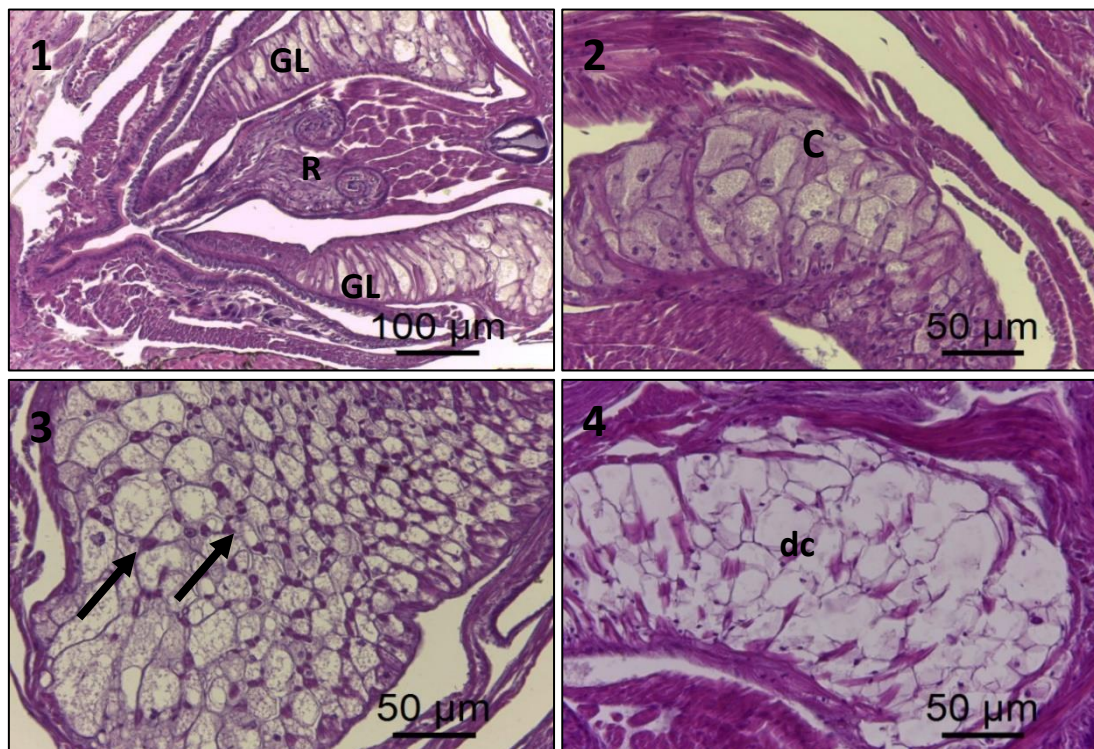
**Fig. 3.1 – 3.6.** Ganglia nervous tissues of *Physa acuta* snails exposed to different concentration levels of NiO ENPs. 3.1 – 3.2 Normal histology: neurons cell bodies (CB); neuropil (NP); 3.3 – 3.6 histopathology: necrosis of neurons cell bodies (arrows) and neuropil degeneration (stars).



## Glands from the foregut

The glandular structure located in the buccal cavity near the radula is composed of mucous secretory cells (Fig. 4.1 and 4.2). The extension of histopathological lesions observed in this organ weren't significantly different between NOEC and LOEC and even in the highest concentration the glands were not extensively affected (Table 3). An inflammatory reaction with haemocytes infiltration (Fig. 4.3) and in more severe cases the occurrence of mucous secretory cells degeneration was detected (Fig. 4.4).

The inflammatory reaction had a more extended response for the lowest concentration of NiO ENPs, while degeneration of mucous secretory cells was the most important histopathological event observed in the snails exposed to the highest concentration (Appendix III – Fig.8). Diffuse (class 3) degeneration of mucous secretory cells affected several snails both in NOEC and LOEC, also the percentage of snails diffusely affected increased with the increase on NiO ENPs concentration.



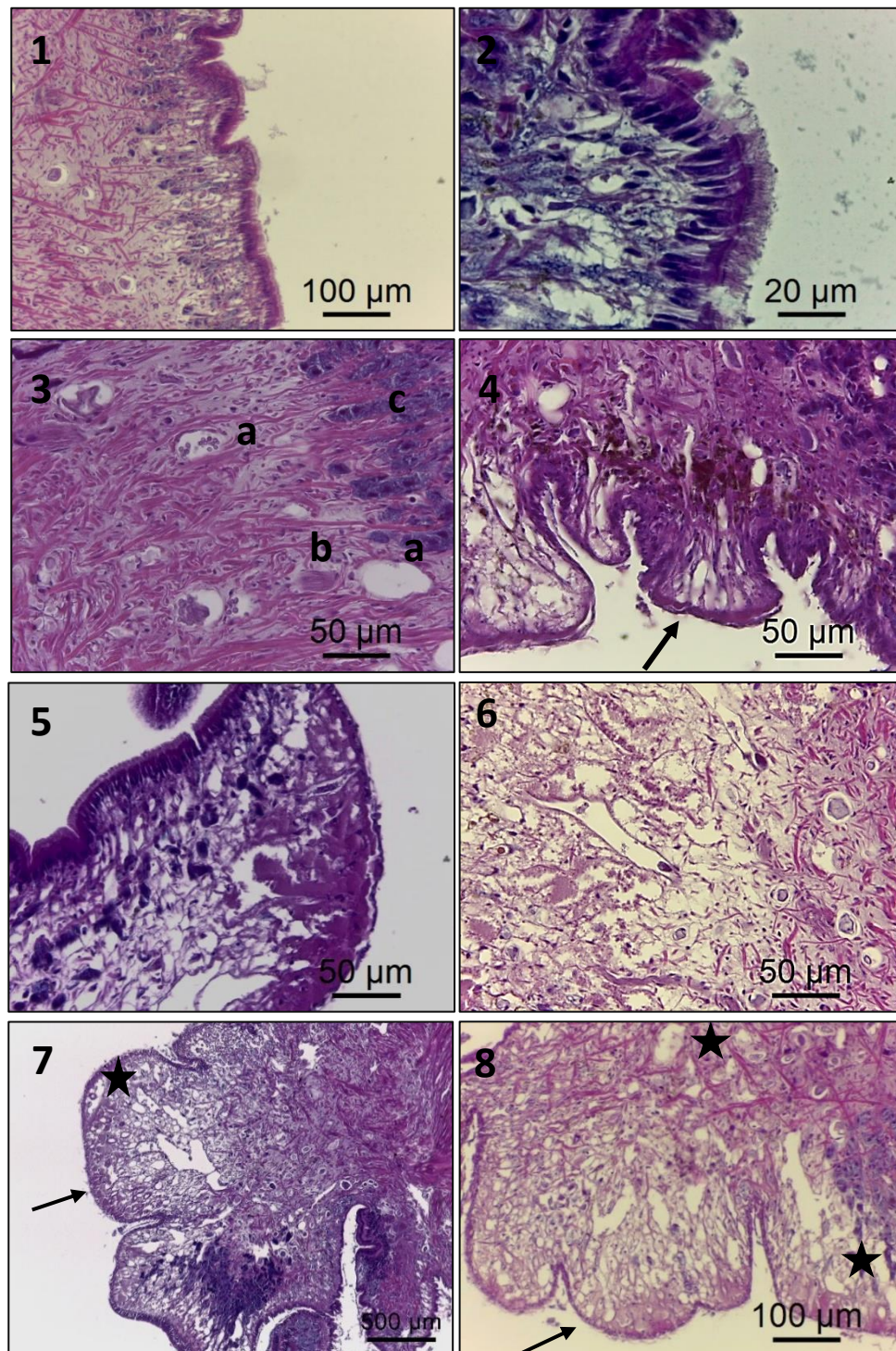
**Fig. 4.1 – 4.4.** Gland from the foregut located near the radula of *Physa acuta* snails exposed to different concentration levels of NiO ENPs. 4.1 – 4.2 Normal histology and location: gland located near radula in the buccal cavity (GL); radula (R); mucous secretory cells (C); 4.3 – 4.4 histopathology. 3 – inflammatory reaction of connective tissue (arrows); 4 – degeneration of the mucous secretory cells (dc).



## Foot

The snail's foot has a simple cylindrical-ciliated epithelium, followed by a dermis with numerous mucous cells, hemocoel spaces, nerves and multiple muscular fibres interspersed in connective tissue (Fig. 5.1 – 5.3). In this organ the extension of the lesions was dose dependent (Table 3). Histologically, the alterations found in the snails' foot were an inflammatory response characterized by increase of pigment deposits (probably melanin) (Fig. 5.4) and haemocyte infiltration (Fig. 5.5), hypertrophy of haemocoel spaces (Fig. 5.7; 5.8), necrosis of foot epithelium (Fig. 5.4; 5.5; 5.7; 5.8), necrosis of adjacent dermis (Fig. 5.7; 5.8) and necrosis of muscle fibres (Fig. 5.5; 5.6).

The extension of the histopathological changes detected in the foot were dose dependent (Table 3). The histological responses which affected higher percentages of snails with severe/diffuse lesions were hypertrophy of haemocoel spaces, necrosis of foot epithelium, necrosis of adjacent dermis and haemocyte infiltration (Appendix III – Fig.9). Also, the lesions that scored severe/diffuse extension (class 3) in NOEC were hypertrophy of haemocoel spaces, necrosis of foot epithelium and necrosis of adjacent dermis (Appendix III – Fig.9). Necrosis of muscle fibres was the less frequent pathology, with diffuse lesions (class 3) only in LOEC (Appendix III – Fig.9). The inflammatory response was more pronounced through the haemocyte infiltration (Appendix III – Fig. 9).



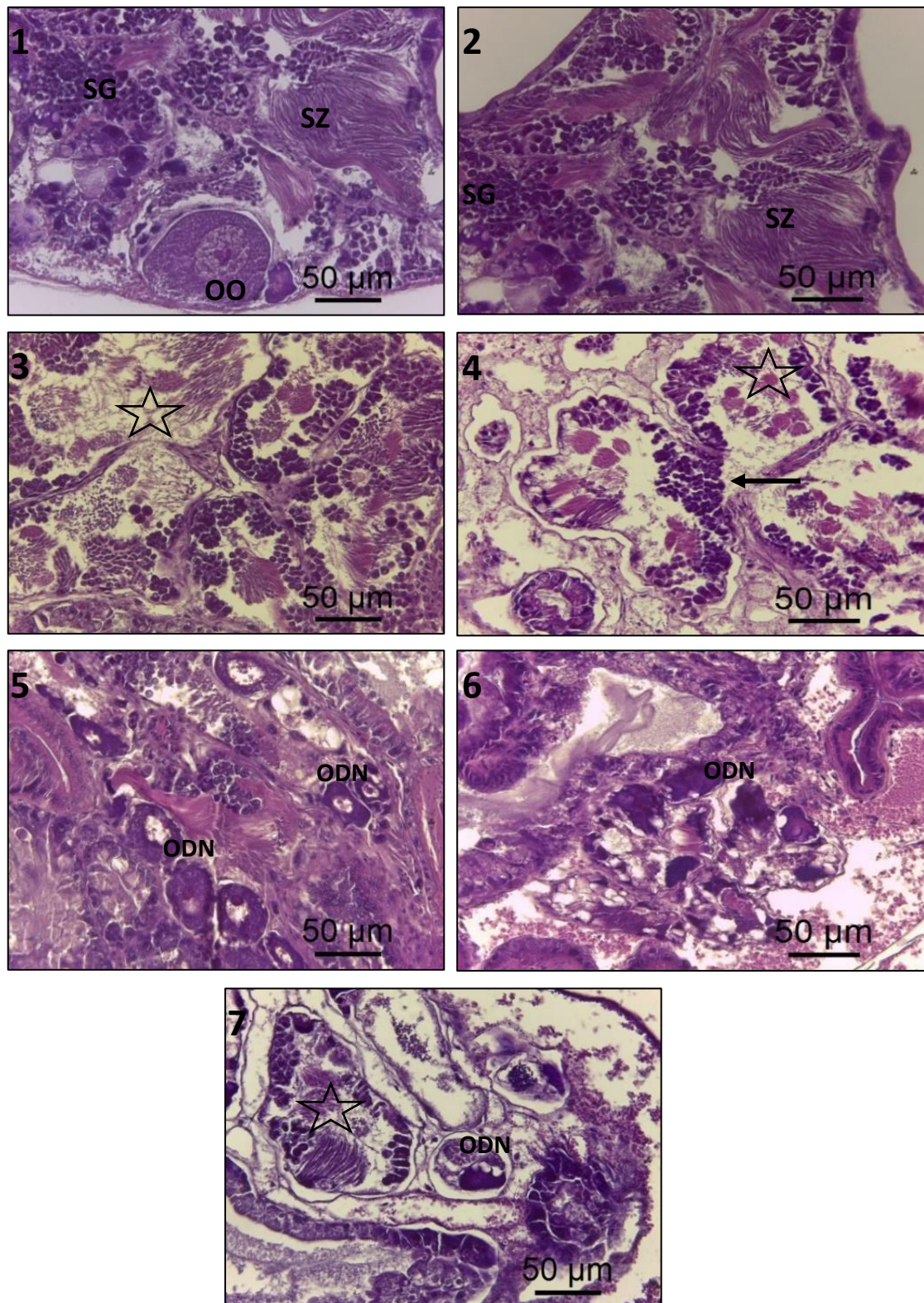
**Fig. 5.1 – 5.8.** Foot of *Physa acuta* snails exposed to different concentration levels of NiO ENPs. 5.1 – 5.3 Normal histology, 5.4 – 5.8 histopathology. 1 – Ciliated epithelium; adjacent dermis and mucous layer; 2 – Detail of cilia; 3 – Detail of haemocoel spaces (a), nerves (b), muscle fibres and mucous cell (c); 4 – Pigment deposits; 5 – Haemocyte infiltration; 6 – Necrosis of muscle fibres; 7 and 8 – Hypertrophy of haemocoel spaces (stars); necrosis of foot epithelium (arrows); necrosis of adjacent dermis; necrosis of muscle fibres.

## Hermaphroditic Gland, Gonad or Ovotestis

Hermaphroditic gland, gonad or ovotestis are the terms used by different authors to refer the organ where male and female gametes are produced. This organ has several follicles surrounded by a follicular epithelium that produces both male and female gametes. When mature, the oocytes occur in the periphery while spermatozoa aggregates towards the centre (Fig. 6.1, 6.2). The extension of histopathological changes observed in the hermaphroditic gland were significantly different from the control only at high levels of NiO ENPs, this is at LOEC (Table 3). The histopathologies found were spermatogonia atrophy and spermatozoa and oocyte degeneration (Fig. 6.3 – 6.7).

The most frequently detected pathologies were spermatozoa and oocyte degeneration. These presented mild, moderate and diffuse histological lesions, even in control groups, but the distribution of affected snails moved to more severe classes as the NiO ENPs concentration increased (Appendix III – Fig. 10). Spermatogonia atrophy had the highest percentage of non-affected (class 0) snails in control and severe/diffuse extensions of lesion was only observed in LOEC (Appendix III – Fig. 10).





**Fig. 6.1 – 6.7.** Hermaphroditic gland of *Physa acuta* snails exposed to different concentrations of NiO ENPs. 6.1 – 6.2 Normal histology: oocyte (OO); spermatogonia (SG); spermatozoa (SZ). 6.3 – 6.7 Histopathology: spermatogonia atrophy – arrows; spermatozoa degeneration – stars; oocyte degeneration – ODN.

## Generalised inflammatory response

A generalised inflammatory response<sup>1</sup>, characterised by haemocyte infiltration and interstitial pigment deposits (probably melanin), was detected in the snails' tissues. This histopathological response was the second more severe and the extension of the lesions was dose dependent (Table 3).

A few snails from control groups showed both haemocyte infiltration and interstitial pigment deposits, but the vast majority presented lower extensions when compared with the groups subjected to NiO ENPs. The extension of histological lesions increased as the NiO ENPs concentration increased. Haemocyte infiltration was the histopathological response more frequently detected with a higher number of snails affected with very extended lesions (the most severe classes) (Appendix 12 – Fig. 11).

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<sup>1</sup> General inflammatory response in all tissues except in the snails' foot. The inflammatory response of the foot was analysed with the data of this tissue.

**Table 3**

Histopathological changes in *Physa acuta* snails' organs (G.N.T. – ganglia nervous tissues; G.F.R. – glands from the foregut located near radula; F. – foot; H.G. – hermaphroditic gland; G.I.R. – generalised inflammatory response in all the body tissues except the foot) according NiO ENPs concentration exposure. Significance accepted when  $p < 0.05$ , according to Jonckheere-Terpstra test. Similar letters indicate non-significative differences.

	0.0 mg/L (Control)	65.3 mg/L (NOEC)	114.3 mg/L (LOEC)	Significance
<b>G.N.T</b>				
Median (mean $\pm$ sd)	0.0 (0.3 $\pm$ 0.5)	1.5 (1.4 $\pm$ 0.8)	3.0 (2.6 $\pm$ 0.7)	p=0.000
range	0.0 – 1.5	0.0 – 2.5	1.0 – 3.0	
n	14	14	14	
	a	b	c	
<b>G.F.R.</b>				
Median (mean $\pm$ sd)	0.8 (0.7 $\pm$ 0.6)	1.5 (1.4 $\pm$ 0.6)	1.5 (1.3 $\pm$ 0.6)	p=0.014
range	0.0 – 1.5	0.0 – 2.5	0.0 – 2.0	
n	10	11	13	
	a	b	b	
<b>F.</b>				
Median (mean $\pm$ sd)	0.2 (0.5 $\pm$ 0.4)	1.2 (1.3 $\pm$ 0.7)	1.7 (1.8 $\pm$ 0.5)	p=0.000
range	0.0 – 1.2	0.5 – 2.3	1.2 – 2.8	
n	15	14	14	
	a	b	c	
<b>H.G.</b>				
Median (mean $\pm$ sd)	1.0 (1.2 $\pm$ 0.6)	1.6 (1.5 $\pm$ 0.5)	1.7 (1.9 $\pm$ 0.7)	p=0.003
range	0.3 – 2.3	0.5 – 2.3	1.0 – 3.0	
n	12	8	14	
	a	a	b	
<b>G.I.R.</b>				
Median (mean $\pm$ sd)	0.5 (0.6 $\pm$ 0.6)	2.5 (2.0 $\pm$ 0.7)	2.5 (2.6 $\pm$ 0.5)	p=0.000
range	0.0 – 1.5	0.5 – 3.0	1.5 – 3.0	
n	15	14	14	
	a	b	c	

## 4. DISCUSSION

Considering the results obtained in the present study, the aquatic gastropod *Physa acuta* is a sensitive organism to NiO ENPs acute exposure and the histological analysis of some organs and tissues of this snail are a promising tool as a biomarker in ENPs toxicological studies.

In this study, the nervous ganglia tissues presented a high sensitive to NiO ENPs, even in sublethal concentrations, showing a severe and extensive histological response. In gastropods the nervous system is composed of ganglia tissue centralized in a nerve ring (Hickman et al., 2011). The nervous tissue is responsible for receive and respond to external and internal stimuli, integrate information and command important motor behaviours (Chase, 2000). Considering this important biological function and the histopathological response found, it is possible that NiO ENPs exposure affected the overall health state of the whole organism.

The glands from the foregut near the radula were not extensively affected. However significant histological alterations also occurred in the sub-lethal concentration of NiO ENPs showing a high sensitivity of this organ. The cell degeneration observed could be due to either necrosis or induced apoptosis since these glands are holocrine. If the histological change observed is due to apoptosis this can be explained by a phenomenon of hyper-secretion induced by the presence of NiO ENPs.

The foot was the organ where the most diverse histopathological phenomena were detected and one of the organs where the extension of the lesions were dose dependent. Most of the histological responses observed in the foot are located near the sole which indicates that NiO ENPs can exert toxicity by contact. The literature reports some histological alterations comparable to the ones found in our work on the foot tissues of aquatic snails when exposed to several chemical compounds. Czech et al. (2001) work demonstrates epithelial alterations (loss of cilia and disorganised layers) and severe inflammatory response in *Lymnaea stagnalis* when chronic subjected to endocrine modulating substances (tributyltin, b-sitosterol, 4-nonylphenol), although the response was time and dose dependent. Cengiz et al. (2005) studied the histological effects of an acute and a chronic exposure to a commercial pesticide in the freshwater snail *Galba truncatula* and reported disruption on muscle fibres, alterations in the number of mucous and protein cells, also dose and time dependent. Although some of the results of these

studies can be comparable with ours we did not find significant alterations in the number of mucous or protein cells.

The hermaphroditic gland is the only organ that presented significative differences in the extension of the lesions just for the higher concentration tested. All the histological changes observed in the gametes, both male and female, can compromise the reproductive success of the species, once the survival of the mollusc populations is closely related with their ability of produce high numbers of offspring. Musee et al. (2010) conducted a study with ENPs on *P. acuta* and reported that sub-lethal concentrations of  $\alpha$ -alumina and  $\gamma$ -alumina causes several defects regarding hatchability and embryo development. Bacchetta et al. (2002) found that the herbicide Paraquat caused not only a significant reduction in the total number of eggs and egg masses, but also histological alterations in the female portion of the hermaphroditic gland on *Physa fontanalis*. In fact, the histological results obtained on the oocytes are very similar to the results obtained in our work, degeneration occurred in all stages of oogenesis even in control groups. As referred by Bacchetta et al. (2002), oocyte degeneration is a commonly observed phenomenon in molluscs that can be provoked by a variety of chemical and physical alterations. But the high and significant percentage of snails extensively affected in the groups exposed to the highest concentration of NiO ENPs indicate that this compound at least promotes the increase of oocyte degeneration. Moreover, our study revealed histological alterations in the male germ lines which is a rare occurrence among toxicological studies regarding aquatic snails, being the more relevant the one conducted by Bighiu et al. (2017) on a dimorphic snail.

The generalised inflammatory response detected in our work was dose dependent and one of most pronounce histological phenomena. These results are in accordance with the inflammatory effects described in the literature for others ENPs in vertebrates (Gojova et al., 2007; Gojova et al., 2009; Kennedy et al., 2009). Additionally, it is described that metal oxide ENPs, including NiO, have the potential to generate free radical activity and the accumulation of reactive oxygen species (ROS) (Hussain et al., 2005; Lu et al., 2009). The inflammatory response is one of the first mechanisms of the innate immune system against injuries, both in vertebrates and invertebrates. In invertebrates, such as gastropods, the melanization and the agglutination (aggregation or clotting) of the circulating fluid are the mains components of the defence system (Buchmann, 2014). Briefly, the melanin and related pigments have not only a defensive role, but also the ability to work as a searcher of ROS and protect the cells and tissues from toxic effects of the free radicals (Edelstein, 1971 in Roberts, 2012; Grimaldi et al.,



2012). At the same time, haemocyte infiltration has a significant part both in defence and healing processes (Sminia et al., 1973). Thus, the important role of pigment deposits, allied to the involvement of haemocyte infiltration in defence and healing processes can explain the substantial inflammatory response found in *P. acuta* snails.

In light of our results, the histopathological responses observed in the studied organs and tissues and the occurrence of a generalized inflammatory response in aquatic snails *P. acuta* have potential as biomarkers of effect due to exposure to ENPs (or, at least, to NiO ENPs).

The main challenges during this work were the lack of quality reference studies on histology and histopathology of gastropods and the technical difficulties of processing the snails. Toxicological studies in aquatic snails use histology of the digestive gland, also known as hepatopancreas, as a biomarker of effect, for its role in detoxification processes (Cengiz et al., 2005; Dummee et al., 2015). However, our work revealed that this organ is an inadequate biomarker because of its quick and high degradation rate, showing frequent and severe alterations even in control groups. Carriker & Bilstad (1946) recommend the injection of the fixative in the snail's pericardium, to prevent the rapidly enzymatic activity that occurs in the digestive gland, but this procedure is not described by many authors and in this case, was not possible due to the small size of the snails. Another technical difficulty found was related with the sectioning of the paraffin blocks. During the processing technique optimization several artefacts were found and, in some cases, the sectioning of the organism wasn't possible. Although sand substrate is widely used in bioassays with aquatic gastropods, to provide conditions similar to the natural environment, this can pose problems when sectioning the specimen. The sand not only got trapped in the snails mucous, but was also ingested by the snails and remained in the digestive tube. Despite the difficulties referred above, and with the exception of the digestive gland, good quality histological slides were obtained, even though the removal of shells and dissection of the posterior intestine to remove the sand was very time-consuming. In the future, addressing these technical issues would allow the standardization of more time-efficient protocols.

Even though the histological alterations near the foot sole may indicate that the NiO ENPs remained in the sediment, one of the questions that arises is if the effects observed were caused by de NiO ENP or if was caused by any dissolution of the Ni in the water. Excessive quantities of Ni in aquatic animals can provoke deleterious health effects including, among others, histopathological damage and oxidative stress (Blewett & Leonard, 2017; Topal et al., 2017). Likewise, and as previously referred, ENPs,

especially metal oxide compounds, can elicit similar effects. Wang et al. (2016) investigated the ions release in aqueous media of several ENPs and although NiO ENPs did not dissolved, it is referred that the dissolution of metal oxide NPs is influenced by many factors including the media composition. Further investigation is needed to understand if the toxic effects found in *P. acuta* are caused by the NiO ENPs, the dissolved Ni or even a combination of both. With this purpose, and the intent of continuing this research, water samples from each one of the tested media were preserved for further analysis.

## 5. Conclusion

The aim of the investigation was to assess the histopathological effects of acute exposure to nickel oxide nanoparticles in the aquatic gastropod *Physa acuta*, also considering the potential usefulness of histology as a biomarker of toxicity.

The findings from this study clearly indicate that the histology of several snails' organs and tissues were affected by NiO ENPs, even in sublethal concentrations. All histopathological effects were observed at NOEC and LOEC and the extension of the lesions was dose dependent in most of the cases.

Furthermore, the interpretation of the histopathological phenomena provides evidence that the contamination route for NiO ENPs in *Physa acuta* were mainly via contact. In this regard, the histological analysis demonstrated to be a promising tool as a biomarker of effect for studies with engineered nanoparticles in the aquatic environment, as well as the use of the aquatic gastropod *Physa acuta*.

It is hoped that these results will contribute to the understanding of the toxic effects of ENPs in the aquatic environment and help to establish new protocols for the control and protection of the aquatic organisms.

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## Appendix I

**Table 4**  
 Histological technique protocol.

Histological technique	
<b>Fixation</b>	
10% buffered formalin	24 h
Alcohol 70%	> 24 h
<b>Dehydration</b>	
Alcohol 70%	3 h
Alcohol 80%	1 h
Alcohol 95%	1 h
Absolute alcohol I	2 h
Absolute alcohol II	2
Xylene I	1 h 30 min
Xylene II	2 h
<b>Impregnation</b>	
Paraffin I	2 h
Paraffin II	2 h
<b>Inclusion</b>	
<b>Sectioning</b>	
<b>Collage</b>	
<b>Drying in oven at 40°C</b>	
<b>Deparaffinisation</b>	
Xylene I	2 min.
Xylene II	2 min.
Absolute alcohol + Xylene (1:1)	2 min.
<b>Rehydration</b>	
Absolute alcohol	2 min.
Alcohol 95%	2 min.
Alcohol 85%	2 min.
Alcohol 75%	2 min.
Washing in tap water	5 min.
<b>Staining</b>	
<b>Dehydration</b>	



<b>Alcohol 85%</b>	1 min.
<b>Alcohol 95%</b>	2 min.
<b>Absolute alcohol</b>	3 min.
<b>Absolute alcohol + Xylene (1:1)</b>	3 min.
<b>Diaphanization</b>	
<b>Xylene</b>	3 min.
<b>Mounting</b>	

## Appendix II

**Table 5**

Haematoxylin and eosin staining protocol.

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### **Haematoxylin and Eosin Staining**

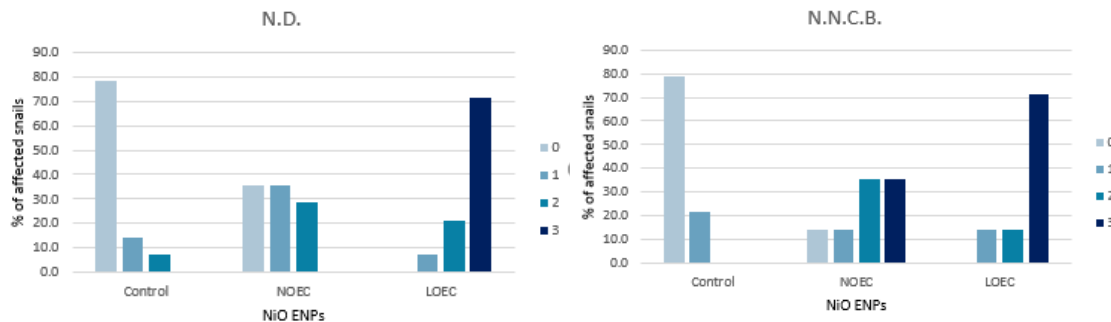
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After deparaffinising and hydrating the sections:

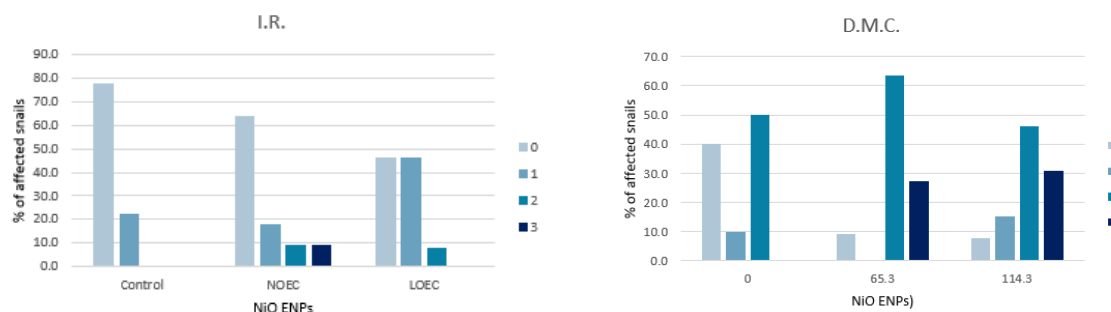
- 1 - Immerse the sections in haematoxylin (Staining I) for 3 minutes.
  - 2 - Wash in tap water for 5 minutes.
  - 3 - Immerse in bluing reagent for 30 seconds.
  - 4 - Wash in tap water for 30 seconds.
  - 7 - Immerse in eosin (Staining II) for 3 minutes.
  - 9 - Dehydrate, diaphanise and mount.
-

## Appendix III

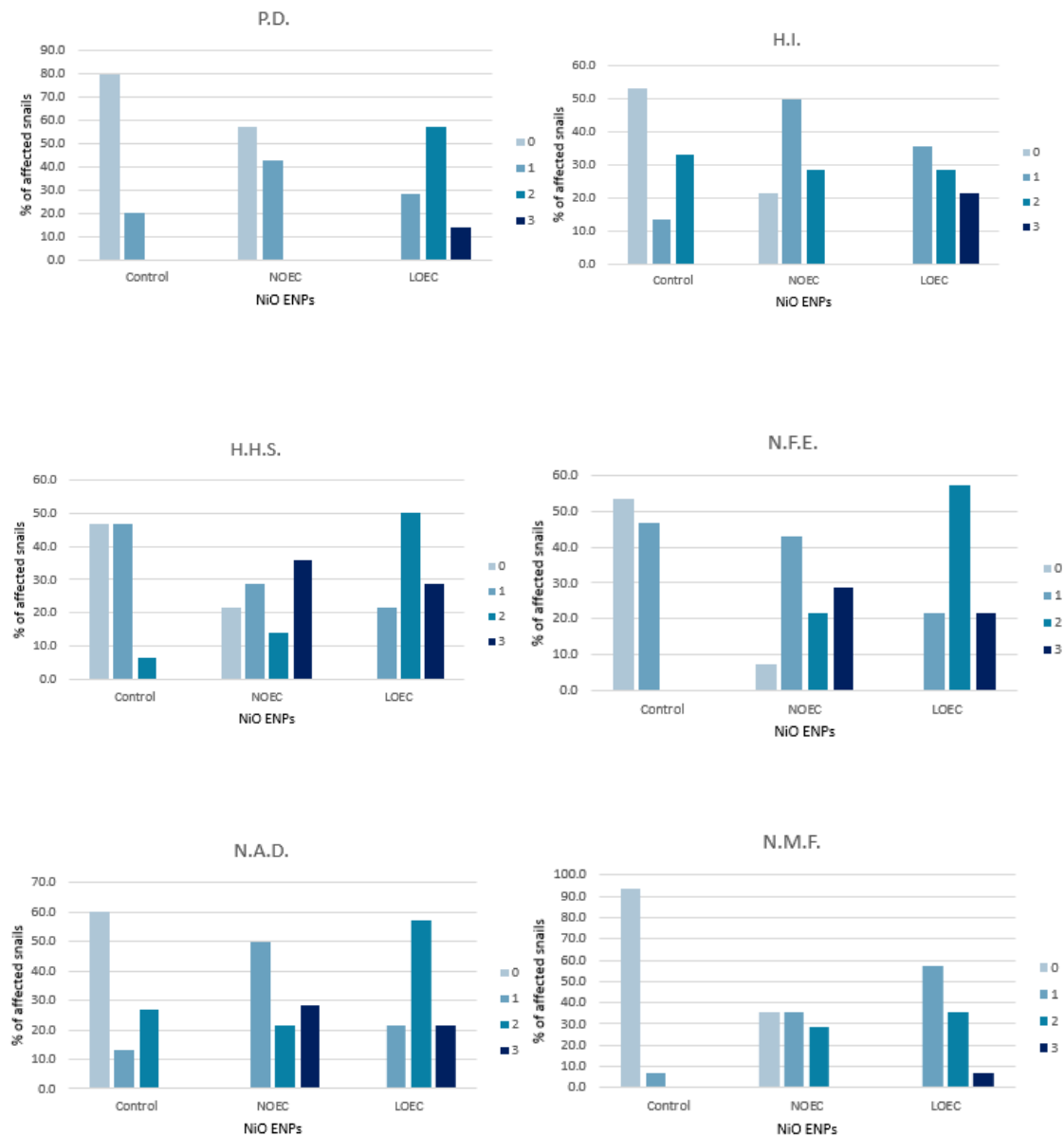
Variation of the extension of histopathological lesions observed in *Physa acuta* organs and tissues exposed to different concentration levels of NiO ENPs (Fig. 7 – 11).



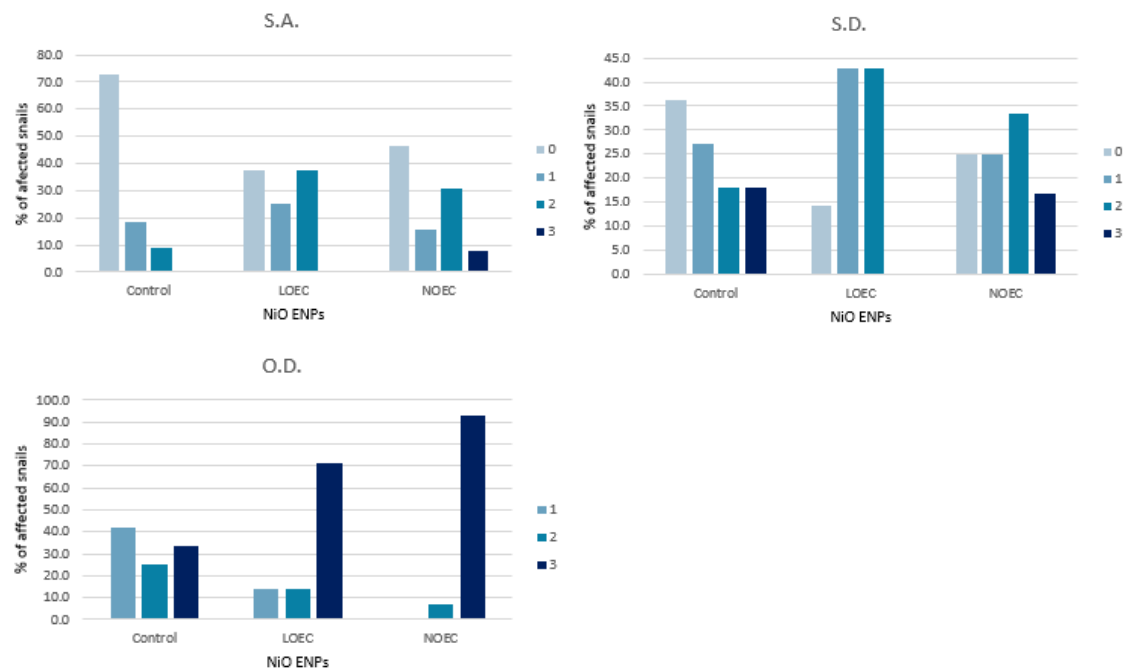
**Fig. 7.** Variation of the extension (0 – unchanged, 1 – mild, 2 – moderate, 3 – severe/diffuse) of histopathological lesions (N.D. – neuropil degeneration; N.N.C.B. – necrosis of neurons cell bodies) observed on ganglia nervous tissues of *Physa acuta* snails exposed to different concentration levels of NiO ENPs (control 0.0 mg/L; NOEC 65.3mg/L; LOEC 114.3 mg/L).



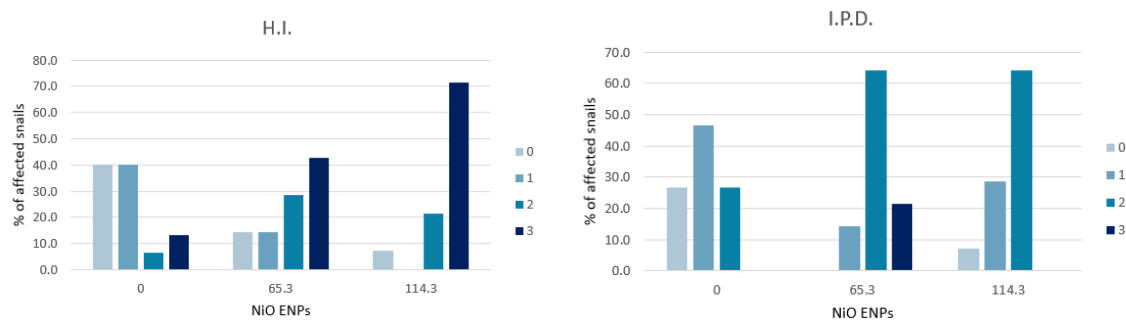
**Fig. 8.** Variation of the extension (0 – unchanged, 1 – mild, 2 – moderate, 3 – severe/diffuse) of histopathological lesions (I.R. – inflammatory reaction of connective tissue; D.M.C. – degeneration of mucous cells) observed on the glands from the foregut located near the radula of *Physa acuta* snails exposed to different concentration levels of NiO ENPs (control 0.0 mg/L; NOEC 65.3mg/L; LOEC 114.3 mg/L).



**Fig. 9.** Variation of the extension (0 – none, 1 – mild, 2 – moderate, 3 – severe/diffuse) of histopathological lesions (P.D. – increase of pigment deposits; H.I. – haemocyte infiltration; H.H.S. – hypertrophy of haemocoel spaces; N.F.E. – necrosis of foot epithelium; N.A.D. – necrosis of adjacent dermis; N.M.F. – necrosis of muscle fibres;) observed on the foot of *Physa acuta* snails exposed to different concentration levels of NiO ENPs (control 0.0 mg/L; NOEC 65.3mg/L; LOEC 114.3 mg/L).



**Fig.10.** Variation of the extension (0 – none, 1 – mild, 2 – moderate, 3 – severe/diffuse) of histopathological lesions (S.D. - spermatozoa degeneration; S.A. - spermatogonia atrophy; O.D. – oocyte degeneration) observed on hermaphroditic gland of *Physa acuta* snails exposed to different concentrations of NiO ENPs (control 0.0 mg/L; NOEC 65.3mg/L; LOEC 114.3 mg/L).



**Fig.11.** Variation of extension (0 – none, 1 – mild, 2 – moderate, 3 – severe/diffuse) of generalized inflammatory response (H.I. – haemocyte infiltration; I.P.D. - interstitial pigment deposits) observed on tissues of all body except in the foot of *Physa acuta* snails exposed to different concentrations of NiO ENPs (control 0.0 mg/L; NOEC 65.3mg/L; LOEC 114.3 mg/L).